Bruce E. Panneton. BEHAVIORAL RESPONSE TO ULTRASOUND STIMULATION AND TYMPANAL CHARACTERIZATION OF *Megacephela carolina carolina* L. (Coleoptera: Carabidae). (Under the direction of Dr. Hal J. Daniel) Department of Biology, May 2002.

The behavioral response of the tiger beetle, *Megacephela carolina carolina* L. to intense ultrasound stimulation was studied. These tests utilized a fixed 40 kHz, 150 dB signal to represent and simulate, generically, the echo locating calls of insectivorous bats, which are potential predators of the insect. The tests were designed to: 1) determine if the insect could detect intense ultrasound, 2) investigate the acoustic startle response (ASR) behavior of the insect and 3) determine the location of the tympanal hearing organs associated with the ultrasound detection—through ablation techniques and subsequent light-microscopic and scanning electron microscopic investigation. Results revealed the presence of tympanal hearing organs on the first abdominal tergum. The insect does respond to intense ultrasound stimulation with a host of ASR behaviors, which suggest the insect has evolved hearing organs as a direct result of the hunting pressures exerted by insectivorous bats.

BEHAVIORAL RESPONSE TO ULTRASOUND STIMULATION AND TYMPANAL CHARACTERIZATION OF *Megacephela carolina carolina* L. (COLOEPTERA: CARABIDAE).

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BEHAVIORAL RESPONSE TO ULTRASOUND STIMULATION AND TYMPANAL CHARACTERIZATION OF *Megacephela carolina carolina* L. (COLOEPTERA: CARABIDAE).

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DEDICATION

This thesis is dedicated to my little brother, Brad. It is my hope that the mere existence of this finished product and achievement will light the path to his success in both life and academics. *There is a light at the end of the tunnel Brad and this book is the proof!*

ACKNOWLEDGEMENTS

I would first like to thank my loving wife, Laura, for her support during this project. Her help in the field, in the lab and on the drawing board were instrumental in the completion of this research. Her dedication to and support of my endeavors is the reason this document has come to be. *With all my love...*

I would also like to recognize my parents, Bruce and Melody, for their never-ending enthusiasm and support of my pursuits, in both life and academics.

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CHAPTER I

THE PROBLEM

The purpose of this study was to investigate the behavioral responses of the tiger beetle *Megacephela carolina carolina,* Linneaus (Coleoptera: Carabidae) to intense ultrasonic stimulation, similar to the stimulation created by echolocating bats (Microchiroptera). This study also documents the external region and morphology of the tympanal hearing organs involved in the detection of ultrasonic signals.

Statement of the Problem

The sound detecting mechanisms of *M. carolina* was first documented on museum specimens of the insect by Spangler in 1988. Ultrasound-tuned tympanal hearing organs have been observed in many different species of insects: mantids, moths, crickets, lacewings and butterflies, as well as beetles. Although the location of the hearing organs vary with each insect, the tympanal hearing organ consists of three fundamental features that are conserved throughout the class: 1) an area of thinned cuticle, known as the tympanum (the focus of this study), 2) an air filled sac or tracheal expansion behind the cuticle; and, 3) a chordotonal sensory organ. The tympanal organs of *M. carolina* are located on the first abdominal tergum under the elytra and wings. Tests were performed to determine if the insects respond to intense ultrasonic stimuli of 40kHz at sound pressure levels in excess of 100 dB (re 20 μPa).

Insects able to detect the ultrasonic emissions of insectivorous bats employ a host of behavioral responses to the sounds enabling them to increase their chances of avoiding aerial predation. These defensive, behavioral responses to ultrasonic stimulation have been collectively labeled as Acoustic Startle Responses (ASR). Typical ASR behaviors include: body posturing, head rolls toward the sound source, abdominal contractions and flight pattern changes. These responses can vary from species to species, yet they seem to remain highly conserved in the Insecta. The acoustic startle response of *M. carolina* to intense ultrasonic stimulation will be explored and discussed. Identification of acoustic startle responses will be accepted as an indication that the tiger beetles can detect intense ultrasonic signals.

Importance of the Study

Hearing organs, utilized for predation avoidance, have evolved at least six separate times in invertebrates. Additionally, there are countless numbers of insects that employ hearing organs for conspecific communication. This research studies the behavioral response of a carabid beetle to intense ultrasound stimulation. The ultrasound stimulation, used in this study, is intended to represent (generically) the echolocation calls of insectivorous bats. This study aids in our understanding of predator-prey interactions, invertebrate behavior and invertebrate bioacoustics and communication.

The results of this research impact many different disciplines, by bringing forth new questions as well. There are questions regarding the evolution of the tympanal hearing organs in *M. carolina*. There are questions regarding the potential for conspecific, ultrasonic communication in *M. carolina*. There are, also, questions regarding the nocturnal behavior of *M. carolina*. Many of these questions will be posed and addressed later in this work.

CHAPTER II

REVIEW OF THE LITERATURE

The literature reviewed for this investigation begins with ultrasound and bat echolocation, followed by the food habits of microchiropteran bats. Next, the literature review will discuss tympanal hearing organs and acoustic startle response (ASR) behaviors in invertebrate insects, concluding with a description of the test subject: the carabid beetle, *Megacephela carolina carolina,* L.

Ultrasound sensitive hearing organs have been identified in members of five insect orders, including the Coleoptera. The other represented orders are: Lepidoptera, Neuroptera, Orthoptera and Mantodea. Evidence suggests that the "ears" of these insects have separately evolved in response to the predation pressures imposed by insectivorous bats that use echolocation while hunting their prey in the night skies (Hoy, 1992; Hoy and Robert, 1996). These bats (Microchiroptera) use ultrasonic sonar to map out their aerial environments and detect potential prey items, like moths (Roeder and Treat, 1961; Roeder, 1972; Spangler, 1988b; Connor, 2001), lacewings (Miller, 1970; May, 1991), crickets (May and Hoy, 1990), mantises (Yager and May, 1990), scarab beetles (Farris, 1994; Forrest et al., 1995; Forrest et al., 1997) and tiger beetles (Spangler, 1988a; Pennisi, 1996).

Ultrasound and Echolocation

Echolocation is a highly specialized form of perception, utilizing neural translation of high frequency echoes to map nocturnal environments where vision is limited. The employment of ultrasound, as opposed to audible sound, is very significant in that ultrasound allows the sender to receive very detailed "glimpses" of its environment, important when navigating the nocturnal skies. A flying bat must recognize and avoid large obstacles such as trees and structures and also recognize small airborne items—its prey. There are about 900 known species of bats worldwide (Neuweiler, 2000); about 750 of these 900 species are known as Microchiroptera (small bats). All Microchiroptera use echolocation to perceive their surroundings and/or hunt for prey (Neuweiler, 2000).

Echolocating bats emit high frequency pulse-emissions from their oral and nasal cavities (Hoy, 1992; Neuweiler, 2000). These emissions ("clicks") are short pulses of high frequency sound ranging from 20-200 kHz (Hoy, 1992) at sound pressure levels that can exceed 100 dB (re 20 μPa) (Griffin, 1984). These intense, high frequency emissions allow bats to identify objects by size, shape and even minute details of surface texture (Neuweiler, 2000).

Echolocation is limited, when compared to visual perception under normal circumstances. Vision, the neural translation of reflected light, gives animals a continuous image flow of their environments. The translation of ultrasonic echoes, however, gives pulsed images similar to the visual effects of a strobe light (Neuweiler, 2000). Echolocation is also limited to the physical constraints of high

frequency sound and the circumstances of its usage. Ultrasonic echoes are usually focused in the direction of flight and do not translate into broad, panoramic images. In humans, these acoustic images would be more closely associated with the visual effects of wearing blinders or tunnel vision.

Echolocation signals range from 80 to 110 dB (re 20 μPa) with average ranges usually less than 20 meters and maximum ranges (open-space search signals) between 50 and 60 meters (Neuweiler, 2000). This range limitation is caused by two different phenomena. The first is image processing and subsequent behavioral responses. In order for a bat to receive a single "image" of its environment it must generate a sound and wait for the corresponding echo. Continuous and timely echo translation is critical to avoiding obstacles and targeting prey.

The second range limitation is based on the physics of ultrasound, instead of functional practicality. Ultrasound attenuates rapidly through geometric attenuation and absorption (Lawrence and Simmons, 1980). Geometric attenuation means that sound pressure levels decrease with distance from the sound source. In free space and ideal conditions without obstacles, sound pressure levels are decreased by ½ for every doubling of distance from the sound source (Ewing, 1989). The attenuation of ultrasound is compounded further by other factors such as sound absorption, which increases with increasing sound frequency (Lawrence and Simmons, 1980).

With all the known limitations to ultrasound and echolocation, it becomes interesting to ponder how bats evolved these abilities. That is, until you put these abilities into their naturally occurring context. When compared to vision, echolocation seems somewhat impotent and useless, until you take away the benefits of light. By evolving a means of perception that does not require daylight to function, echolocating bats were able to take over a niche (the nighttime skies) dominated by an abundant food source (invertebrates). Additionally, no bats are blind. If fact, many bats have excellent vision. Visual cues are believed to be used by bats for orientation and for discerning darkness to determine the appropriate times to exit and return to their roosts before and after feeding (Whitaker and Hamilton, 1998).

Coleopterans as a Food Source for Insectivorous Bats

Bats are top-level predators in the night skies. According to McCracken (1986) 20 million Mexican free-tailed bats *Tadarida brasiliensis* from one Texas cave can consume 125 tons of insects in a single night. Eastula and Whitaker (1972) found that the diets of seven species of insectivorous bats, found in the Big Bend National Park of Texas, ranged from 6.4 to 44.4% Coleopterans (mostly Carabid and Scarab beetles). The only other order that consistently outranked the beetles was Lepidoptera (moths and butterflies).

Coleoptera is the most specious order in the animal kingdom (Wilson and Peter, 1989; Booth et al., 1990) with over 300,000 known species (Papp, 1984).

There are 20 species of bats in the eastern United States (Whitaker and Hamilton, 1998). Nineteen of these 20 species are insectivorous. Of these 19 species of bats, 13 are known or suspected to include beetles in their diets and have habitat ranges that overlap with the known distribution of *M. carolina*, the focus of this study (Knisley and Shultz, 1997; Whitaker and Hamilton, 1998) see Table 1 (potential predator listing) and Figure 1 (*M. carolina* distribution).

Vespertilionid, or Mouse-Eared bats account for almost all of these 19 potential coleopteran predators and is the largest family of bats in the world with 318 species worldwide and 14 species in the eastern United States (Whitaker and Hamilton, 1998). All mouse-eared bats are insectivorous and have highly developed echolocation abilities. This evidence shows the high level of predation pressures that can be exerted by the bats on beetles. Thus it stands to reason that many nocturnal beetles, including *M. carolina*, would have developed and evolved ultrasound sensitive hearing structures, similar to other documented cases in other orders of insects, in order to increase their survivability.

Tympanal Hearing Organs and Acoustic Startle Response

Insects appear to detect ultrasonic echolocation signals with tympanal hearing organs (May, 1991; Hoy, 1992; Hoy and Robert, 1996). Tympanal ears are characterized by three morphological features: 1) an area of thinned cuticle (tympanal membrane) backed by 2) an air-filled sac or tracheal expansion that allows the membrane to resonate to sound induced pressure changes, this

resonation stimulates 3) an associated chordotonal sensory organ whose scolopoid bodies terminate in cap cells (Ewing, 1989; Hoy and Robert, 1996). Scolopoid bodies are individual sensory units within the chordotonal organ. These organs are associated with the nervous system and work in conjunction with the axons and dendrites in the delivery of information to the insect brain. Scolopodia can range from a few to hundreds of units are responsible for the insects' ability to detect a wide range of sound frequencies (Fullard and Yack, 1993). Fewer scolopodia leads to a more narrow range of hearing sensitivity (Ewing, 1989). See Figures 2 and 3 for a generic schematic of a scolopiod body and tympanal hearing organ.

Tympanal organs are pressure receivers. Pressure receivers, by definition, require a small air space to allow for resonation. Tympanal hearing organs are believed to have evolved from small tracheal expansions within the insect cuticle (Blum, 1985). The chordotonal sensory organs associated with these insect "ears" have been described by Blum (1985) as modified vibrational sensilla. Tympanal organs we see today could have evolved from proprioceptors (integumental sensory organs) that may have been adjacent to the ancestral tracheal expansions mentioned above. These sensilla were most likely stimulated by the sound pressure-induced resonation of the air space within the expansions, which could have prompted some of the earliest forms of Acoustic Startle Response (ASR) behaviors (Blum 1985).

According to Hoy and co-workers (1989), tympanal hearing organs have evolved, separately, in at least 6 different orders of insects. These tympana have

been found in many unique places in different species, yet most of the organs are tuned to very similar frequency ranges (20-80 kHz), which fall well within the ultrasonic frequency ranges of foraging bats. Roeder (1972) found that some sphingid moths have tympana in their mouthparts and respond to acoustic stimuli between 30 and 70 kHz. Green lacewings (*Chrysopa carnea*) have tympanal hearing organs in their forewings (Miller, 1970). Some species of crickets have tympanal organs in their prothoracic legs and respond to ultrasonic acoustic stimuli between 30 and 90 kHz (Moiseff et al., 1978; May, 1991; Hoy and Robert, 1996). Yager and co-workers (1990) found that some mantids have tympana between their metathoracic legs and respond to ultrasonic stimulations between 20 and 60 kHz. Some scarab beetles have ultrasonic sensitive tympana in the cervical membrane, that are acoustically tuned to frequencies between 20 and 80 kHz (Forrest et al., 1995; Forrest et al., 1997). Many tiger beetles (Cicindellidae) have tympana in their first abdominal tergum and respond to acoustic stimuli of 40kHz (Spangler, 1988a) see Table 2 for a listing of insects that utilize tympanal hearing organs for predator avoidance. Evidence suggests that the ears of these insects have evolved in response to the predation pressures imposed by insectivorous bats that use echolocation while hunting their prey in the night skies (Hoy, 1992).

Different insect orders have evolved diverse yet similar hearing structures to presumably detect the ultrasonic output of echolocating bats. This hearing ability has led to the evolution of many stereotypical behavioral responses. This ASR

behavior has been documented in Coleoptera, Lepidoptera, Neuroptera, Orthoptera and Mantodea. Typical ASR behaviors include: body posture changes, flight pattern changes, abdominal contractions, stop responses, head rolls toward the sound source and leg placement changes (Spangler, 1988a; Hoy et al., 1989; Ewing, 1989; Yager et al., 1990; May, 1991; Hoy, 1992; Farris, 1994; Forrest et al., 1995 and Forrest et al., 1997).

***Megacephela carolina carolina*, L.**

Tiger beetles, the focus of this research, have been found to have tympana acoustically tuned to ultrasonic frequencies (Spangler, 1988a). The research of Spangler (1988a) and Yager and Spangler (1995, 1997) has shown that many *Cicindella* species have tympana broadly tuned to ultrasonic frequencies. The sensitivity of the organs is greatest around 40 kHz (between 30-60 kHz) with behavioral thresholds averaging 75-80 dB (re 20 μPa). The basic ASR behavior of the studied tiger beetles, when immobilized, are abdominal contractions (Spangler 1988a). Other, more advanced ASR behaviors were found in *Cicindella marutha*. These advanced behaviors include aerodynamic flight changes and even sound production believed to be designed to "jam" or distort the echo that a foraging bat would receive, thus aiding in the insects' escape (Yager and Spangler, 1997).

Although the majority of tiger beetle ASR research has been conducted on the more common *Cicindella* species, some mention has been made about the elusive

and understudied *M. carolina.* In his 1988 publication, Spangler noted that museum specimens of *M. carolina* have enlarged tympana that are:

> ...close together at the midline of the tergum and expand both anteriorly and posteriorly, suggesting an adaptation that would increase sensitivity to lower frequency sound.

M. carolina is very easily distinguished from members of the Cicindella. Barry Knisley and Tom Schultz, in The Biology of Tiger Beetles and a Guide to the species of the South Atlantic States, (1997) describe this insect as:

> ...body length is 12-20mm. The dorsal surface is dark metallic green...The ventral surface is metallic green to dark bluish-black with the posterior abdominal sternite and lateral portions of the subterminal sclerites yellow-white to cream colored. Legs, antennae and mouth parts are light brown... (see Figure 4 for a schematic of *M. carolina* and Plate 1 for color photograph of adult).

M. carolina is nocturnal and gregarious. They seem to prefer very moist water-edged habitats and can be found during the daylight hours hiding under debris (see Plates 2 and 3). Unlike many diurnal tiger beetles found in the southeast, *M. carolina* does not usually take flight when disturbed. Instead, they run surprisingly fast. Since these beetles are nocturnal and have the ability to fly, they are presumed to be targets of insectivorous bats. Thus, the tympana found on museum specimens by Spangler (1988a) can be presumed to be tuned to ultrasonic frequencies somewhere between 20 and 200kHz and the insects are presumed to use some form of ASR behavioral response to ultrasonic stimulation.

The purpose of the following research is to test these aforementioned presumptions. Tests will be performed to study the behavioral response of *M. carolina* to intense ultrasonic stimulation. Detailed micrographic and photographic representations of the tympanal region will be included and discussed.

Table 1. Potential Predators

Bats with habitat ranges that overlap with *M. carolina*

Scientific Name	Common Name	Notes
Myotis austroriparius	S.Eastern Myotis	Coleopterans = 7.2 - 26.3% of diet
Myotis grisescens	Gray Myotis	Scat Studies have revealed Carabid remains
Myotis septentrionalis	Northern Myotis	Coleopterans are major food source
Myotis sodalis	Indiana Myotis	Coleopterans are major food source
Pipistrellus subflavus	Eastern Pipistrelle	Coleopterans are major food source (18% Carabids)
Eptesicus fuscus	Big Brown Bat	Scat Studies: Coleopterans 36.1% (Hamilton 1933)
Lasiurus borealis	Eastern Red Bat	Coleopterans are major food source
Lasiurus cinereus	Hoary Bat	Coleopterans = 20 - 50% of diet depending on age and skill (Rolseth et al. 1994)
Lasiurus intermedius	Northern Yellow Bat	Coleopterans are major food source
Lasiurus seminolus	Seminole Bat	Coleopterans = 10% of diet in July and **90% of diet in August**
Nycticeius humeralis	Evening Bat	Scat Studies: Coleopterans 60% (Whitaker and Clem 1992)
Corynorhinus rafinesquii	Rafinesque's Big-Eared Bat	Lepidopterans are primary food source: 90%
Tadarida brasiliensis	Brazilian/Mexican Free-Tailed Bat	Coleopterans and Lepidopterans are major food sources

Bats who's habitat ranges have **limited** overlap with *M. carolina*

Scientific Name	Common Name
Myotis leibii	Eastern small-footed Myotis
Myotis lucifugus	Little Brown Bat
Lasionycteris noctivagans	Silver-Haired Bat
Corynorhinus townsendii	Townsend's Big-Eared Bat

Potential Bat predators of *M. carolina* Adapted from Whitaker and Hamilton (1998).

Figure 1: A map showing the distribution of *M. carolina* throughout most of the southern United States—states where *M. carolina* have been collected are shaded. Adapted from Knisley and Schultz, 1997.

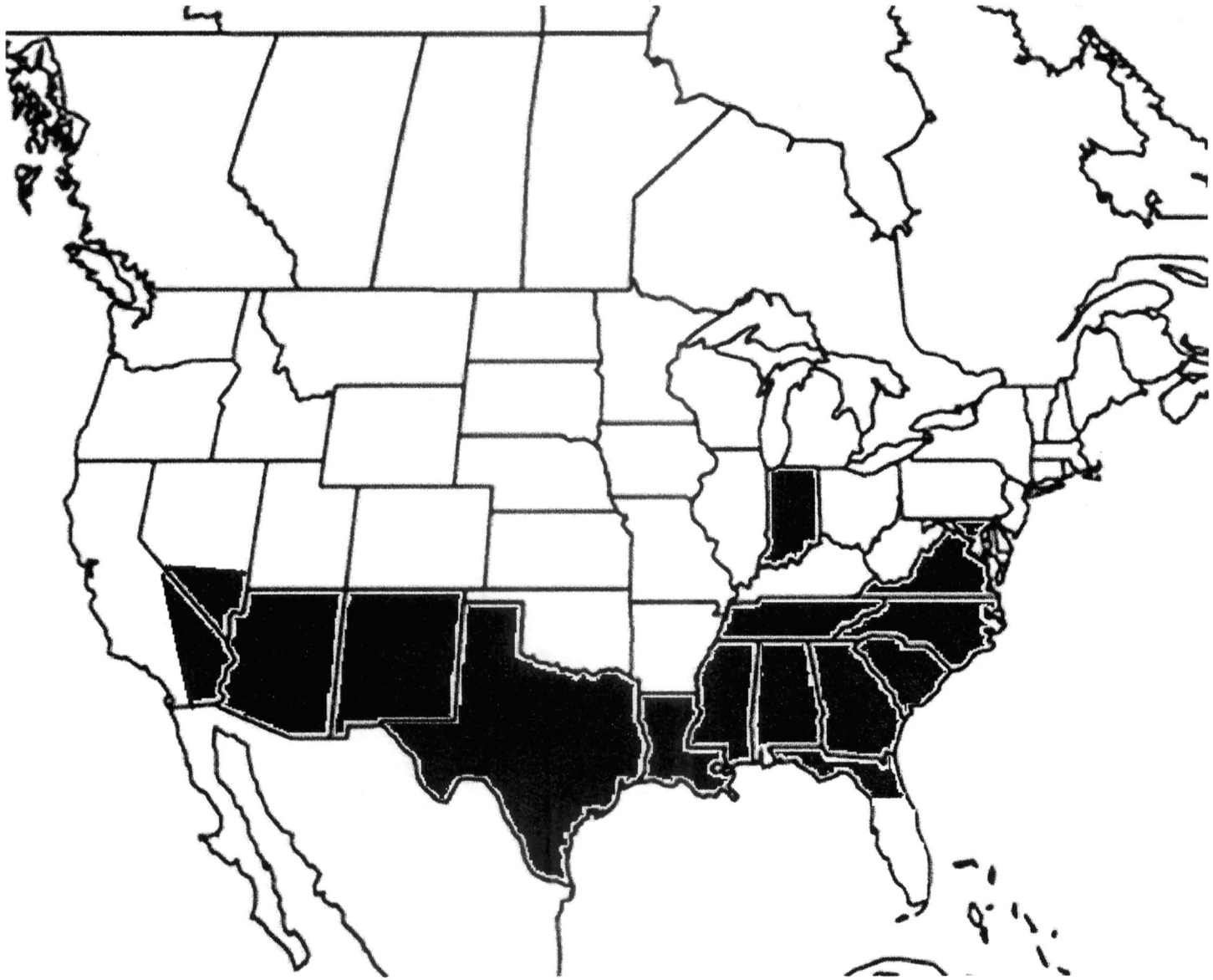
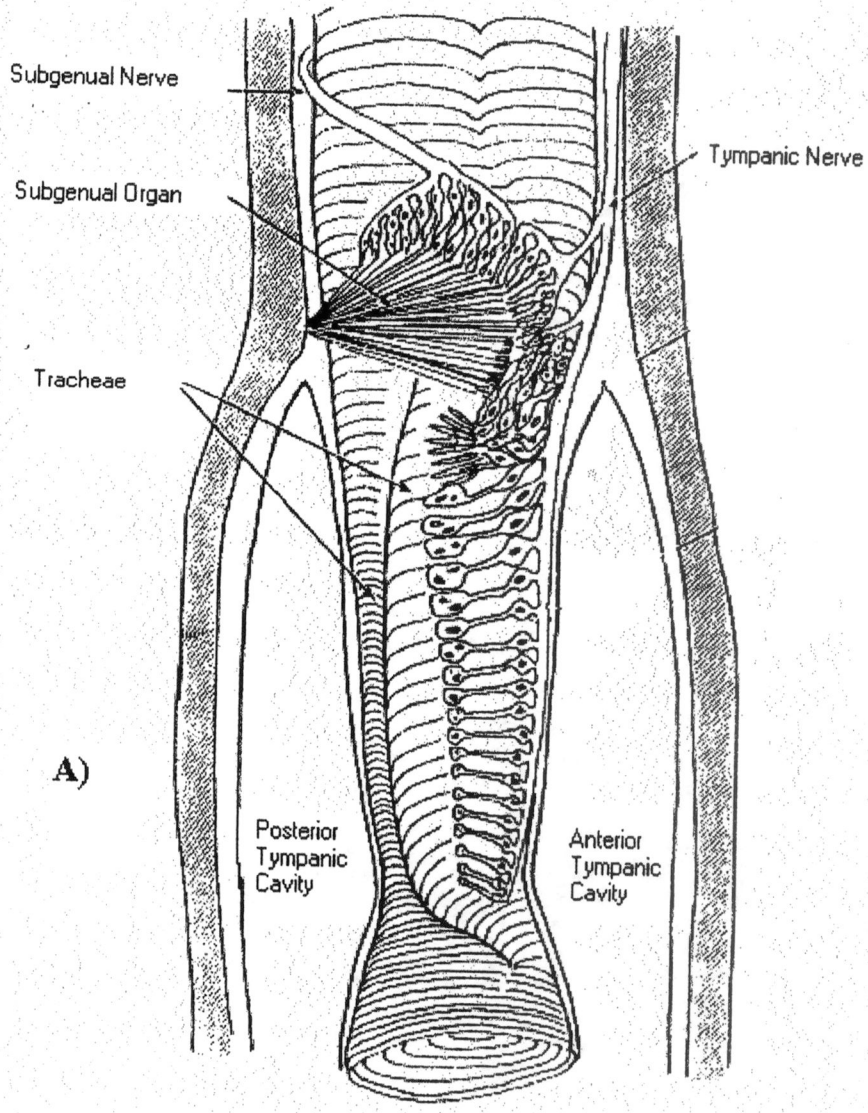


Figure 2 and 3: Schematic structure of the tympanal hearing organ (left) found in the prothoracic leg of a bush cricket and a schematic of a chordotonal sensesillum—scolopoid body (right). These adapted schematics are commonly used diagrams found in many works and adapted, specifically for this literature review, from: Ewing, 1989 (from Schwabe, 1906) and Blum, 1985 (from V.G. Dethier, *The Physiology of Insects*, Methuen, Inc.,1964).


Subgenual Nerve
Subgenual Organ
Tracheae
Tympanic Nerve
A)
Posterior Tympanic Cavity
Anterior Tympanic Cavity

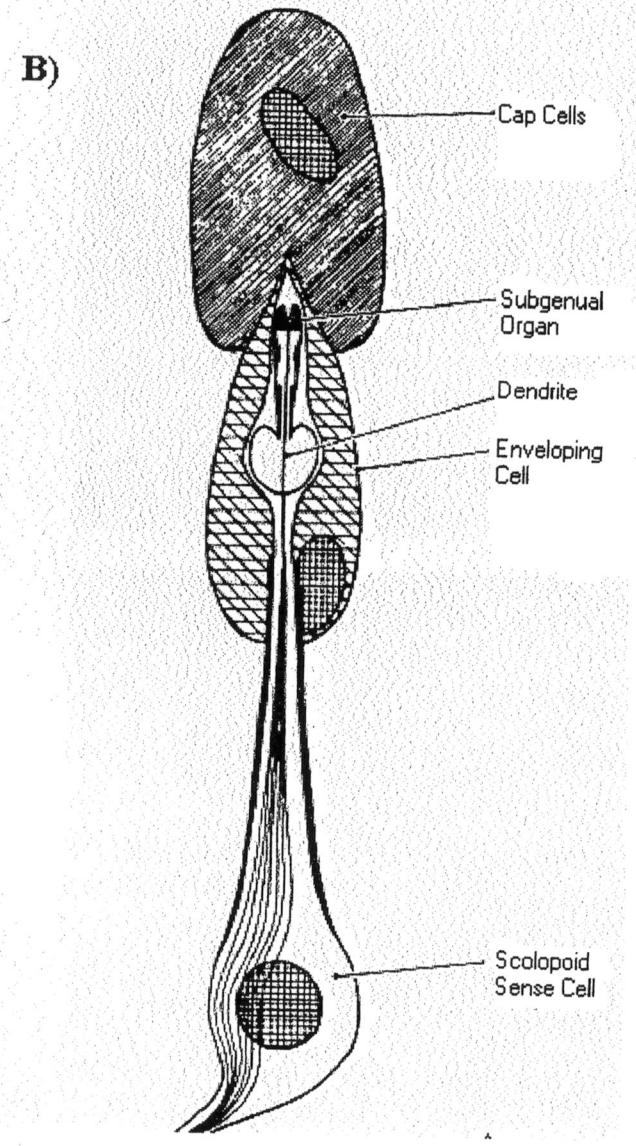

B)
Cap Cells
Subgenual Organ
Dendrite
Enveloping Cell
Scolopoid Sense Cell

Table 2. List of Insects with Tympanal Hearing Organs

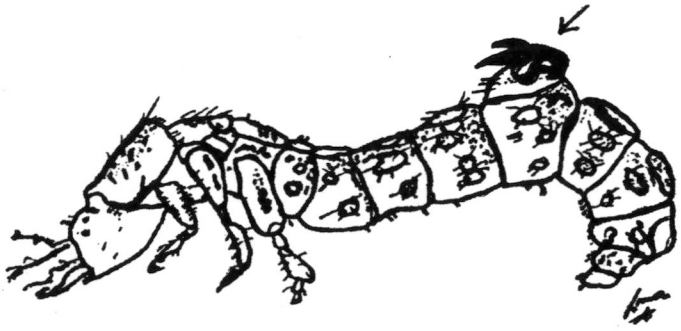
Order	(Family) Genus species	Location	Ultrasound Specs	References
Orthoptera	(Gryllidae) Many *spp.* of cricket	Prothoracic Leg	30-90 kHz	Moiseff et al. (1978)
	(Acrididae) Many *spp.* of Katydid	Prothoracic Leg	*	Hoy RR (1992)
Neuroptera	*Chysopa carnea*	Fore wing	*	Miller LA (1975)
Lepidoptera	(Sphingidae) Many *spp.* of moths	Mouthparts	30-70 kHz	Roeder KD (1972)
Blattodea	*Periplanteta americana*	Ab segments 2-6	*	Florentine GJ (1967)
Mantodea	(Mantidae) *Parasphendale agrionina*	Between Metathor. legs	20-60 kHz @ +64 dB	Yager et al. (1990)
Diptera	*Ormia ochracea*	Behind head	5-20kHz	Narins (2001)
Coleoptera	**(Carabidae)**			
	Cicindella marutha	1st ab segment	40 kHz @ 70-75 dB	Spangler HG (1988), Yager and Spangler (1995)
	Megacephela spp.	"	Museum Specimen	Spangle HG (1988)
	(Scarabaeidae)			
	Euetheola spp.	Behind the head	20-80 kHz @ <70 dB	Forrest TG et al. (1997)
	Cyclocephala spp.	"	ASR @ 40 kHz	"
	Dyscinetus spp.	"	ASR @ 40 kHz	"
	Oxygrylius spp.	"	ASR @ 40 kHz	"

This table shows the many different Orders of insects with documented tympanal hearing organs. As you can see, the 40 kHz signal that used in this study is well within the frequency ranges of nearly all of the previously studied animals and should adequately serve the needs of this study.

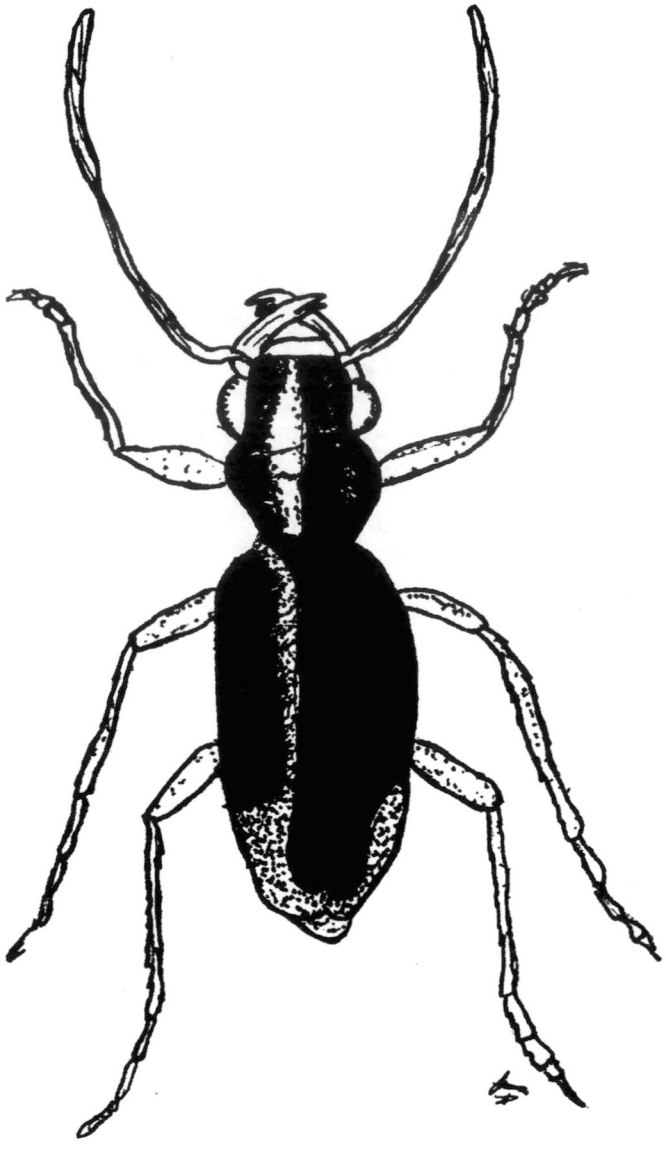
* Specific ultrasound specs were not available

Figure 4: Schematic of *M. carolina* (B) and side profile of a tiger beetle larva (A). Art work by Laura A. Otrimski.

A)



B)



CHAPTER III

MATERIALS AND METHODS

This study examined the presence of tympanal hearing organs in *Megacephela carolina carolina,* L. as well as the behavioral response of the insect to intense ultrasound stimulation. Acoustic startle response behaviors were investigated using repeated, intense, ultrasound stimuli from a hand-held ultrasound generator. Ablation tests were performed to determine the location of the suspected tympanal hearing organs.

Capture and Keeping of Tiger Beetles

All insects were collected between July and September of 1999 and 2000 in Pitt County, North Carolina and surrounding areas. The specimens were collected in low-lying moderately wet areas. *M. carolina* prefers sandy-soiled environments with patchy vegetation and open sandy spaces. The beetles can be found during the daylight hours under bark, wrack, downed tree limbs, tarps and other forms of litter in the areas mentioned above. The insects were captured by hand and kept cryogenically in small compartmentalized containers that were transported in small coolers with frozen ice packs. The extremely low temperatures inside the coolers induced a temporary state of hibernation and kept the insects from escaping every time the lid was opened. The insects were kept on ice until they were taken back to the lab and released into small, plastic aquaria known as "Kritter Keepers®" (manufactured by Lee's—size: Kritter Keeper Large

Rectangle 14 1/2"L x 9 3/4"H x 8 3/4"W). Some of the insects remained in the coolers for over two (2) hours. All of the insects (32) were recovered from the cold transport, in the lab, with no losses.

The aquaria housing the beetles were lined with 1 to 1.25 cm of sand, which was partially covered with moist paper towels. The paper towels and small sections of the sand were moistened daily with a spray bottle application of a 1:4 hydrogen peroxide and water solution. This solution helped to limit fungus and mold growth in the containers. The paper towels served a dual role. First of all, the paper towel served as cover for the insects to hide under during the day. Secondly, the paper towels were used as feeding platforms (see Plate 4). The tiger beetles were fed small portions of lean ground beef every 2-3 days—in the evenings, just before they became active. The food was placed on the paper towels and the paper towels were replaced prior to every feeding in order to keep any mold and fungal growth (on the sand) to a minimum. The aquaria were kept in cool dry places during the majority of day and periodically placed in windowsills to keep the containers from becoming too cool.

Ultrasound Equipment

The instrument used to produce the ultrasonic signal was a Sonin 45 ® ultrasonic tape measure (henceforth referred to as the Sonin45). This hand-held ultrasound generator is designed to give distance measurements up to 45 feet by projecting a burst of ultrasound and receiving its echo and calculating the time

lapsed in between (see Plate 5). The device was tested with a Gunner Rasmussen® model 140BP microphone (flat frequency response 0-100 kHz +/- 3 dB) and analyzed with a Techtronics digital oscilloscope (® Tekscope model THS 710, 60 MHz). A signal was obtained on the oscilloscope and the period of the wave and envelope was measured. The measurements revealed that the device produces a 40 kHz signal with sound pressure levels in excess of 150 dB (re 20 μPa) at 10 cm. This device produces an intense, fixed ultrasonic signal. The frequency of this signal falls well within the frequency range of many insectivorous bats (see Table 1) and is very similar to the sound stimulation levels used by others (Spangler, 1988; Farris, 1994; Yager and Spangler, 1995; Forrest et al., 1997).

Ultrasound Stimulation Tests

The insects were glued and immobilized on the end of a stiff copper wire (12-15cm) with Hotstik® Coolpac© low temperature hot glue. The wire was attached on one end to the thoracic region of the insects in order to keep their head and abdominal areas completely mobile and unhindered during the stimulation trials (see Plate 6). The other end of the wire was connected to a ring stand so that the beetle could be suspended under an Olympus ® SZH stereo-zoom microscope. The microscope and camera equipment was used during the preliminary tests (200 trials) so that the experimenter could ascertain the subtleties of *M. carolina's* ASR behaviors and for documentation. An X-acto® Extra Hands with 2x magnifier,

hands free magnifying glass was used, in place of the microscope, for repeated tests following recognition of the ASR behaviors.

Once the insect was affixed to the wire, the ultrasound tests began. The Sonin45 was held 10 to 15cm from the tethered insect and turned on by depressing the power button. Stimulation lasted 2-4 seconds and was repeated 5 times. Each beetle was tested once per day (five trials per test) every 3-5 days. Testing was performed every 3-5 days, as opposed to every day, in order to try to keep the insects from becoming accustomed to the Sonin45's signal.

The beetles were tested in three different ways. The preliminary tests and first battery of tests were performed on completely intact tiger beetles. The insects were tethered to the copper wire and tested with no physical alterations (32 subjects: 535 trials). The second battery tests were performed on tethered tiger beetles with their elytra and wings removed (18 subjects: 90 trials). The final battery of tests was performed on tiger beetles with complete tympanal ablation (17 subjects: 85 trials).

Tympanal Ablation

To expose the dorsal, abdominal surface of a beetle the elytra and wings must be removed. This delicate process begins with holding the insect between the index finger and thumb. Using forceps, the experimenter grabbed the anal tip of one elytron and turned the wrist slightly out and gently lifted up on the elytra to "unlock" the protective covering. The experimenter then pulled the tip forward,

laterally, until the elytron was in the fully extended position. Once the elytron was forced into its fully extended position it will not return to its "closed" position quickly. At this point, cuticle scissors are used to snip the elytron off at its connection point on the pleural process. After the elytron was removed, the cuticle scissors were used to remove the associated wing. This process is repeated for both elytra and wings. Another method for removing the elytra is by gripping the tip of one elytron with forceps, as before, and forcing the elytron superiorly, similar to opening a soda can. This process is much quicker, less subtle and more destructive to the aforementioned pleural process, which is adjacent to the first abdominal tergum and is therefore not recommended.

In order to render the tympanal region ineffective a 000 insect pin was inserted into the tympanal region of the first abdominal tergum and removed, leaving the tympana ablated. See Plate 12 for an SEM image of an ablated tympanal region.

Acoustic Startle Behaviors

During the acoustic testing, a noticeable behavioral change at the onset of ultrasound stimulation was assured as a positive indication that *M. carolina* could detect intense ultrasound signals. The behaviors of interest were: body posturing, abdominal contractions, head rolls toward the sound source and stop behaviors having all been documented as ASR behaviors in other insects. These behaviors were classified on a graded scale of positive responses, no response (negative) responses and unknown (questionable) responses.

Each tiger beetle was tested 1 to 6 different times depending on the in-lab life span of the individual and the battery of tests being performed. Chi-Square analysis was used to analyze the raw data to retain or refute the following **null hypothesis: There is no statistical difference in the behavioral responses of specimens with intact, suspected, tympanal organs compared to the behavioral responses of specimens with their suspected tympanal organs ablated.**

Characterization of Tympana

Spangler (1988a) made a note in his work about the location and general shape of the tympana of *M. carolina*. The purpose of these tests was to give precise measurements and up-close microscopic documentation of the area of thinned cuticle along the first abdominal tergum of these tiger beetles. Once testing was completed, or an individual specimen died, the insects were placed in small vials of Trump's Fixative, a solution of gluteraldehyde and formaldehyde in a sodium cacodylate buffer. This fixative kills the insects quickly and preserves them until they are to be tested. All scanning electron images were taken on a Phillips® 501B microscope linked to a Gatan Digiscan® and Apple Power Macintosh G4® computer. The specimens were rinsed twice in 0.2 M sodium cacodylate buffer for 5 minutes. Excess water was removed by ethanol series dehydration. The specimens were then dried in a Balzers Union ® CPD 020 critical point drier and sputter coated with gold palladium alloy by a Technics® Hummer V sputter-

coater. All other microscopic images were obtained by an Olympus ® C35 AD-2 35 mm camera and exposure control unit attached to an Olympus ® SZH stereo-zoom microscope.

CHAPTER IV

RESULTS AND DISCUSSION

Capture and Keeping of Insects

In addition to *Megacephela carolina carolina* L., this investigator has observed various species of diurnal tiger beetles. There are very distinct differences between *Cicindella spp.* tiger beetles and *M. carolina,* especially in terms of capturing and maintaining the insects. Capturing *M. carolina* is much different from capturing diurnal *Cicindella spp.* To capture diurnal tiger beetles, a researcher needs a net and quick reflexes along with a little practice. Diurnal tiger beetles are quick to take flight from perceived threats. They usually do not fly very far, but they have a tendency to land several feet from where they took off facing the direction of their perceived threat (personal observation). It is important for a collector to sneak up on diurnal tiger beetles with the sun in his/her face so a shadow doesn't signal the beetle before he/she is close enough take a swipe with the net. Capturing *M. carolina*, on the other hand, requires a little persistence, patience and speed. Since these tiger beetles hide under debris during the day, sneaking around and solar orientation is not required. As soon as *M. carolina* is uncovered the insect will run, with surprising speed, away from the threat. It is important to keep up with the insect and make quick grabs. One should not attempt to gently grab the insect itself. Instead, it is preferable to grab the insect and some of the substrate under it. This method serves a dual purpose. First of all, this method increases the chances of success because instead of trying to grab a

moving insect 12-20 mm long, one can grab a piece of palm-sized earth with the insect in it. Secondly, gabbing soil with the insect decreases the chances of injuring the tiger beetle.

In addition, maintaining *M. carolina,* in the lab is much different from diurnal *Cicindella spp.* Diurnal tiger beetles prefer warm, well-lit laboratory settings. These C*icindella spp.* tiger beetles are extremely active during daylight hours. In a warm, well-lit aquarium it is not uncommon to observe these tiger beetles running too and from cover flying, fighting, mating or eating. Mold and fungus growth on these insects is not as much of a threat because they prefer drier environments. Dehydration, however, is a much greater threat. The water in moist sand evaporates quickly in a well-lit, warm aquarium. Diurnal tiger beetles can dehydrate and die in a matter of 1-2 days in the lab if not watered regularly. Keeping a moist paper towel in the container and soaking one small corner of the aquarium is sufficient to keep *Cicindella spp.* tiger beetles hydrated. *M. carolina* are completely inactive during the day and will stay under cover for the entire day if they are not disturbed. As the sun begins to set, the insects begin to move around in their laboratory aquaria. Because of their preference for lower temperatures, *M. carolina* does not move around quite as fast nor are they as active as their diurnal cousins. Their preference for moist habitats also promotes the growth of mold and fungus, which are both significant threats for these beetles in the lab. Dark fungus or mold growth is very easy to notice on the legs and antennae these tiger beetles because of their light colors. By feeding the insects on

the paper towels, changing the paper towels regularly and hydrating the soil and towels (see Plate 4) with a 1:4 peroxide/water solution, mold and fungus growth can be minimized.

Ultrasound Generator

The Sonin45 is very simple to use and is relatively inexpensive when compared to other acoustic equipment. It is a very effective tool for producing high intensity ultrasound. The Sonin45 produces a fixed 40kHz signal with sound pressure levels in excess of 150 dB (re 20 μPa). Most of the insects studied have SPL behavioral thresholds of 85 dB (re 20 μPa) or less.

This ultrasound generator will continually send pulsed emissions when holding down the activation button. The experimenter has used the Sonin45 at night when bats were flying around a small pond and found that the bats would immediately turn away from the sound if the generator was activated.

During the preliminary tests, the experimenter noticed that some of the tethered beetles would show distinctive behavioral responses to the "clicking" noise made when the activation button on the Sonin45 was pressed. After a few trials, the activation button was depressed under the table before stimulating *M. carolina* so the tethered insect would not hear the "click." With the button depressed the experimenter would bring the apparatus up from under the table and "sweep" the signal by the insect (on varied sides). This method seemed to be more representative of the sweeping search patterns used by bats, as they would

forage for flying insects. This method produced more pronounced and distinctive responses and was used throughout all the tests.

Hearing Trial Results

In the hearing trials with elytra intact, 306 out of the 535 trials (57.2%) were positive responses to ultrasound stimulation. There were 168 negative responses (31.4%) and 61 unknown or questionable responses (11.4%). The trials with the elytra removed revealed 72.2% positive responses (65 out of 90 trials). There were 19 negative responses (21.1%) and 6 unknown or questionable responses (6.7%). In the total ablation trials, with both tympanal regions destroyed, there were no positive responses documented out of 85 trials (0.0%). There were, however, 72 negative responses (84.7%) and 13 unknown or questionable responses (15.3%) to ultrasound stimulation.

Two hundred preliminary (learning) tests were performed in the late summer of 1999. Here the experimenter learned behaviors to look for as positive indications of ultrasound detection. Stop movements and flattening behaviors were the most easily recognized and common behaviors noticed. Abdominal contractions, as documented by Spangler (1988a), were very difficult to detect with these insects.

Stop movements are very easy to recognize. The tethered tiger beetles do one of three things when they are attached to the copper wire: 1) stand in place (motionless); 2) run in place; and 3) sometimes fly in place. Stimulation with

ultrasound when the insect is running in place, causes the tiger beetle to stop and remain perfectly still during the first few moments of a continuous signal. When the insect is flying in place (see Plate 9), ultrasound stimulation causes the beetle to stop the flight behavior all together. This behavior suggests that echolocation detection, while in flight, will cause *M. carolina* to stop flight and drop to the ground.

The flattening behavior is equally interesting. Sometimes when a tethered, motionless tiger beetle is stimulated with ultrasound, it will stretch its legs out as if to flatten its body against the substrate. This behavior suggests that echolocation detection, while the beetle is standing still, will cause *M. carolina* to flatten its body against the ground in an attempt to diminish its echo against its surroundings.

During the subsequent 335 tests, 90 elytra removal tests and 85 ablation tests, the experimenter began to notice subtle behavioral responses to ultrasound stimulation: antennae movements and abdominal movements. Many times the experimenter noticed, in association with stop behaviors, the antennae of the tethered *M. carolina* would move in the direction of the sound and follow its sweeping motion. The abdominal movements were even more pronounced and more easily noticed when the insect was tethered upside-down. Sometimes the insects would move the tip of their abdomens in the direction of the sound and they would also move with the sweeping motion of the sound stimulus. These behaviors were noticed when the sound was introduced from either side and from

behind. These behaviors suggest the insect can determine sound direction and that *M. carolina* strives to orient its body so as to always be facing away from the perceived threat. This behavior is similar to the running behavior exhibited by *M. carolina* to visual threats and is a direct contradiction to the avoidance behaviors exhibited by diurnal Cicindellidae to visual threats in which they fly and land facing the direction of their enemy (personal observation).

These abdominal and antennae movements could also be visual behavioral responses. The tiger beetles could very well be visually perceiving the movements of the Sonin45 (from side introductions) and therefore be trying to run away from the perceived threat. When the stimulus is introduced from behind the insect, chances are the beetle cannot see the Sonin45 and is therefore reacting to the ultrasound itself.

Three hundred and seventy one of the 535 trials (57.2%) indicated positive acoustic startle response behaviors to ultrasound stimulation in intact test subjects. Seventy two percent of 90 trials indicated positive acoustic startle response behaviors to ultrasound stimulation in *M. carolina* with elytra and wings removed. Seventy-two of the 85 trials (84.7%) indicated negative acoustic startle response behaviors to ultrasound stimulation when the suspected tympanal hearing organs were ablated (the insects used in the elytra removal tests were used in the ablation tests 1 day later—one insect died overnight). With intact tympana, *M. carolina* responded to intense ultrasound stimulation 371 out of 625 trials or 59.3%.

Chi-Square analysis revealed a significant difference in the behavioral responses of specimens with intact tympanal organs compared to the behavioral responses of specimens with their tympanal organs ablated ($X^2 = 34.52$, df = 2, $p < .001$).

This evidence suggests that these tiger beetles do indeed have the ability to perceive ultrasonic signals and subsequently do employ a host of Acoustic Startle Responses including: stop behaviors, flattening behaviors, antennae movements and abdominal movements toward the source. With the tympanal regions completely ablated, *M. carolina* did not show positive ASR behaviors in any of the 85 trials. This evidence suggests that the area described by Spangler (1988a) is indeed a tympanal hearing organ.

Characterization of Tympanal Organs

Ablation tests confirm that tympanal hearing organs are present on the first abdominal tergum of *M. carolina* (see Plate 12). Plate 7 represents the external characteristics of the hearing organs. This image shows the larger size of the first tergum, in relation to the remaining tergii. This size relation can also be seen in the SEM image of Plate 10. Plate 8 shows the "swollen" nature of the first abdominal tergum. This "swollen" appearance is a result of the adaptation of tracheal expansions within the tergum. These tracheal expansions are the air-filled resonation chambers of the tympanal hearing organ. In Plate 7 distinct tracheal

expansions are evident on the first abdominal tergum, separated by the midline of the insect.

These dark regions are made evident by areas of thinned cuticle (C), the tympanum, that cover the resonation chambers of the hearing organ. Each tympanum is very smooth and free of hairs, sensilla, denticles or other sclerites normally associated with the abdominal tergii of beetles (see Plate 11). This smoothness and uniformity most likely enables efficient sound detection.

Scanning electron microscopic images and color micrographs were used in studying both live and dead *M. carolina* specimens. Scanning electron micrographs are instrumental in discerning the surface texture of the tympani. SEM images give a clear, up-close, monochrome view of the surface of the tympanal hearing organs of dead specimens. Color micrographs, however, give indications of texture, thickness, color and subsurface cavities of the live specimens. These color images are best for displaying the external anatomy of the tympanal hearing organs for this research.

Table 3. Chi-Square Analysis of Data--Long form with Calculations

Responses to Ultrasound Stimulation*

	Positive	Negative	Unknown	Total
Intact Tympana	371 (389.96)	187 (164.61)	67 (70.42)	**625**
Ablated Tympana	72 (53.03)	0 (22.38)	13 (9.57)	**85**
Total	**443**	**187**	**80**	**710**

* Data in parentheses, of table, are the calculated Expected Values

Degrees of freedom (df) = (rows - 1) x (columns - 1)
df = (2-1) x (3-1) = 2

Calculating expected frequencies for each cell ...

Processing row 1, column 1 ...
Observed value (O) = 371
Expected value (E) = (row total x column total) / grand total
E = (625 x 443) / 710 = 389.964788732394
Chi-square = (O - E) squared / E
Chi-square = ((371 - 389.964788732394) **2) / 389.964788732394
Chi-square = 0.922296633071567
Total chi-square now = 0.922296633071567

Processing row 1, column 2 ...
Observed value (O) = 187
Expected value (E) = (row total x column total) / grand total
E = (625 x 187) / 710 = 164.612676056338
Chi-square = (O - E) squared / E
Chi-square = ((187 - 164.612676056338) **2) / 164.612676056338
Chi-square = 3.04467605633803
Total chi-square now = 3.9669726894096

Processing row 1, column 3 ...
Observed value (O) = 67
Expected value (E) = (row total x column total) / grand total
E = (625 x 80) / 710 = 70.4225352112676
Chi-square = (O - E) squared / E
Chi-square = ((67 - 70.4225352112676) **2) / 70.4225352112676
Chi-square = 0.166335211267605
Total chi-square now = 4.1333079006772

Processing row 2, column 1 ...
Observed value (O) = 72
Expected value (E) = (row total x column total) / grand total
E = (85 x 443) / 710 = 53.0352112676056
Chi-square = (O - E) squared / E

Chi-square = ((72 - 53.0352112676056) **2) / 53.0352112676056
Chi-square = 6.78159289023211
Total chi-square now = 10.9149007909093

Processing row 2, column 2 ...
Observed value (O) = 0
Expected value (E) = (row total x column total) / grand total
E = (85 x 187) / 710 = 22.387323943662
Chi-square = (O - E) squared / E
Chi-square = ((0 - 22.387323943662) **2) / 22.387323943662
Chi-square = 22.387323943662
Total chi-square now = 33.3022247345713

Processing row 2, column 3 ...
Observed value (O) = 13
Expected value (E) = (row total x column total) / grand total
E = (85 x 80) / 710 = 9.57746478873239
Chi-square = (O - E) squared / E
Chi-square = ((13 - 9.57746478873239) **2) / 9.57746478873239
Chi-square = 1.22305302402651
Total chi-square now = 34.5252777585978

Calculating probability (P) ...
Looking up critical values for chi at df = 2:
Sig levels: 0.20 0.10 0.05 0.025 0.01 0.001
Crit vals: 3.22 4.61 5.99 7.38 9.21 13.82
Sig. 0.20: chi is greater than or equal to 3.22
Sig. 0.10: chi is greater than or equal to 4.61
Sig. 0.05: chi is greater than or equal to 5.99
Sig. 0.025: chi is greater than or equal to 7.38
Sig. 0.01: chi is greater than or equal to 9.21
Sig. 0.001: chi is greater than or equal to 13.82

Degrees of freedom: 2
Chi-square = 34.5252777585978
***p* is less than or equal to 0.001.**
The distribution is significant.

Chi-square formatting and calculation proofs were aided by the Web Chi-Square Calculator software developed and employed by the Georgetown University Dept of Linguistics.

www.georgetown.edu/cball/webtools/web_chi.html

CHAPTER V

SUMMARY AND CONCLUSIONS

Summary of the Study

Megacephela carolina carolina L. has a pair of tympanal hearing organs on the first abdominal tergum. Ablation tests confirmed the nature and location of these organs. Ultrasound stimulation tests have confirmed that these organs are acoustically tuned to intense ultrasound and the detection of intense ultrasound elicits Acoustic Startle Response behaviors. Some documented ASR behaviors include: stop behaviors, flattening behaviors, antennae movements and abdominal movements toward the ultrasound source. All of this evidence, in addition to the fact that *M. carolina* is nocturnal and capable of flight, suggests that *M. carolina* has evolved tympanal hearing organs and ASR behaviors as a defensive mechanism against insectivorous bat predation.

There is, however, another behavior worthy of noting. *M. carolina* is capable of flying. The insect has functioning wings and elytra that are not fused. Also, tethered flight behavior has been elicited in the lab. All of this information has been established by experts and witnessed by the experimenter. Also established is the fact that *M. carolina* is not prone to take flight like it's diurnal cousins. Perhaps this adaptation is for predator avoidance as well.

It is speculated that this adaptation has come about as a result of the insect's habitat selection. Tiger beetles seem to fly with a ballistic trajectory. That is, they don't seem to make advanced maneuvers like dipterans or hymenopterans. Taking

defensive nocturnal flight with a ballistic trajectory in a water-edged habitat can lead to life ending landings (in water). Perhaps this behavior is not an adaptation at all, but a limitation due to flight-thermoregulatory requirements. This behavior poses a critical question in understanding the acoustic nature of *M. carolina.*

Why are *M. carolina* tiger beetles less likely to take flight as regularly as diurnal tiger beetles? What is the advantage of being able to detect the ultrasound of bat sonar when the insect exercises non-flight behaviors? These questions can be summed up into one question: What is the purpose of *M. carolina's* tympana and ultrasound sensitivity: communication, prey detection, predator avoidance?

Suggestions for Further Research

The following suggestions for further studies are offered by the investigator:

1. The frequency range and behavioral thresholds of *M. carolina* must be established (both sonic and ultrasonic). This data will provide critical information that will enable researchers to better understand the acoustic nature of the insects. What animals produce sound within those ranges? Are those animals predators or potential prey? Finding the answers to the above questions will help researchers understand for what use *M. carolina* uses its tympanal hearing organs.

2. Another area of suggested research focuses on the acoustic communication behaviors of the insects themselves. Does *M. carolina* communicate with audible sound or ultrasound? If so, how does the insect produce the sound? Is there sexual dimorphism in the insects' ability to detect and/or create sound?

3. Finally, the flight behaviors and preferences of the insect should be studied. This research may help in understanding the nature and purpose of the hearing organs. Is the "flightless behavior" a preference or is it a habitat-specific adaptation? Is *M. carolina* subjected to the hunting pressures of bats? If so, is the flightless behavior and adaptation to avoid bat predation or are the hearing organs actually used for prey detection or conspecific communication?

Plate 1: Color Photograph of *M. carolina* adapted from Knisley and Schultz (1997). Photograph by T. Koenig, 1994.

Plate 2: Color Photograph of *M. carolina* habitat.


T. Koenig 1994

Plate 3: Color photograph of *M. carolina* habitat substrate. Note the overturned debris (tree branch) and litter (plastic bottle). During the day, *M. carolina* would be found hiding under these items.

Plate 4: Color photograph of *M. carolina* lab aquaria. Note the folded paper towel that served as a feeding platform, moisture reserve and cover for the specimens.

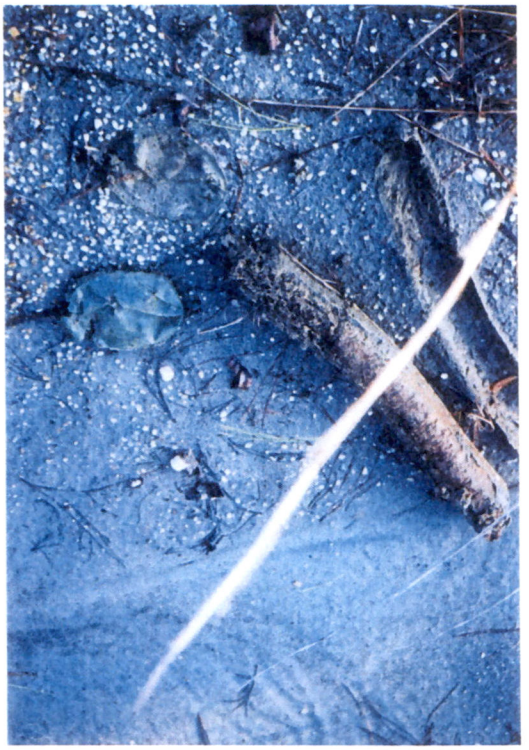
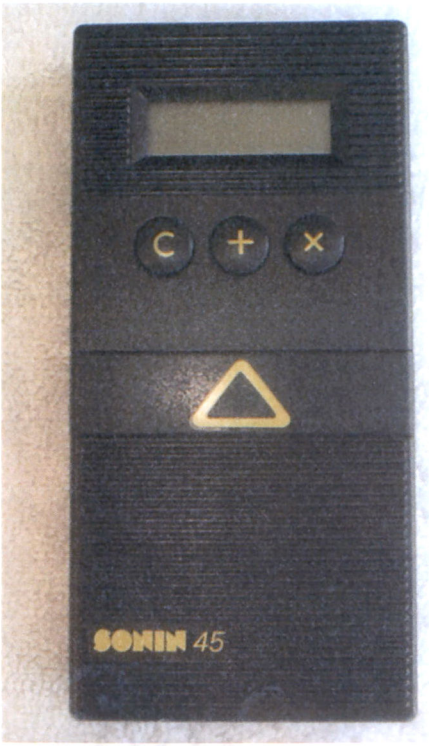


Plate 5: Color Image of the Sonin® 45 Ultrasonic Tape Measure used as the ultrasound generator.

Plate 6: Micrograph of tethered *M. carolina*. All insects were tested under these conditions. Note the attachment point of the wire tether (W) along the thoracic region. This method of attachment is non-invasive, removable (low temperature hot glue (G) is easily removed) and allows for unrestricted movement of the head, legs and abdomen. 5X


C
+
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SONIN 45

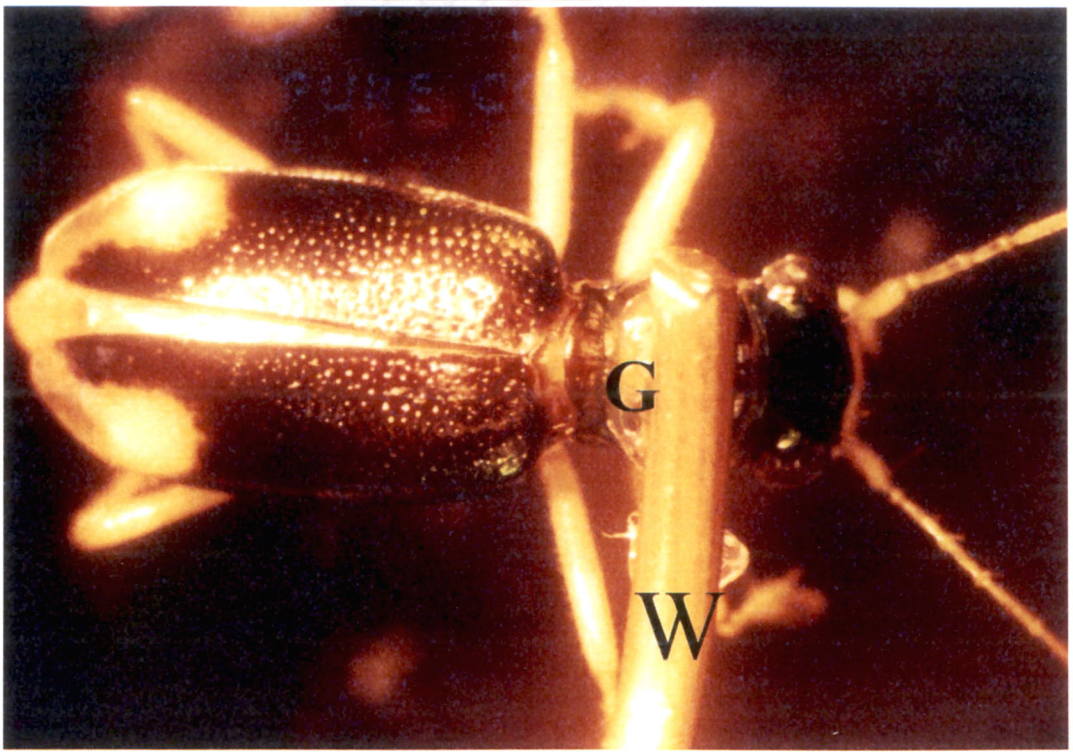
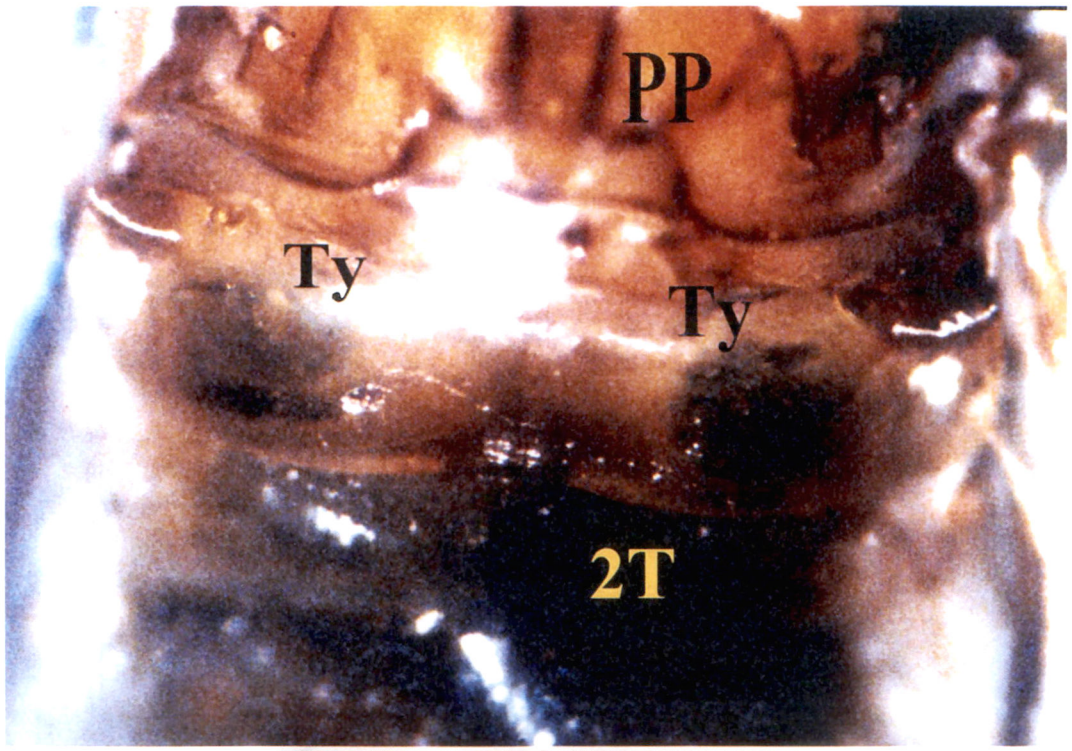

G
W

Plate 7: Micrograph of the first abdominal tergum and tympanal regions (Ty) of a live *M. carolina* tiger beetle. Note the two dark "spaces" within the first abdominal tergum. These dark spaces are areas of thinned cuticle © backed by the air filled resonation chambers of the tympanal hearing organs. The pleural process is labeled (PP) and the second abdominal tergum is labeled (2T). 10X

Plate 8: Micrograph of the right tympana (Ty) of a live *M. carolina* tiger beetle. The pleural process is labeled (PP). Again, note the larger size of the 1st abdominal tergum in relation to the other 2 tergii in the picture (2T and 3T). The larger size of the 1st abdominal segment allows for the air-filled, resonation chamber of each tympanal organ. Note the area of thinned cuticle (dark spot to the right of "Ty" label) along the first abdominal segment. This area is the tympana. 15x.


PP
Ty
Ty
2T

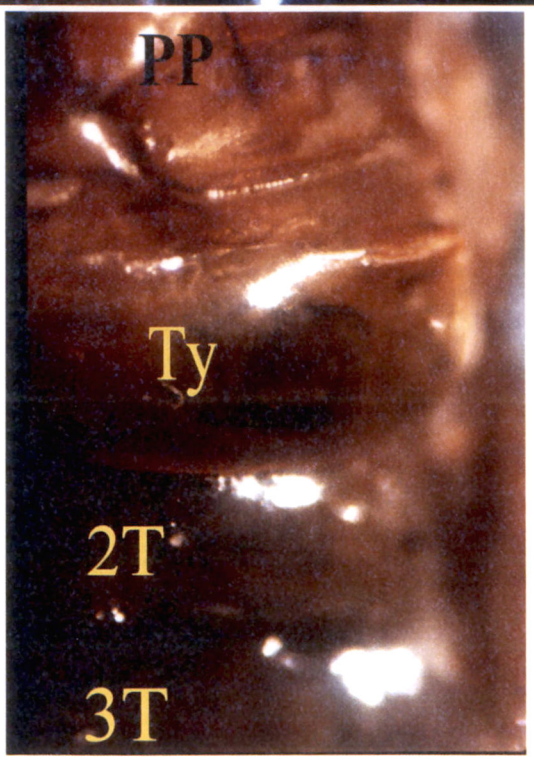
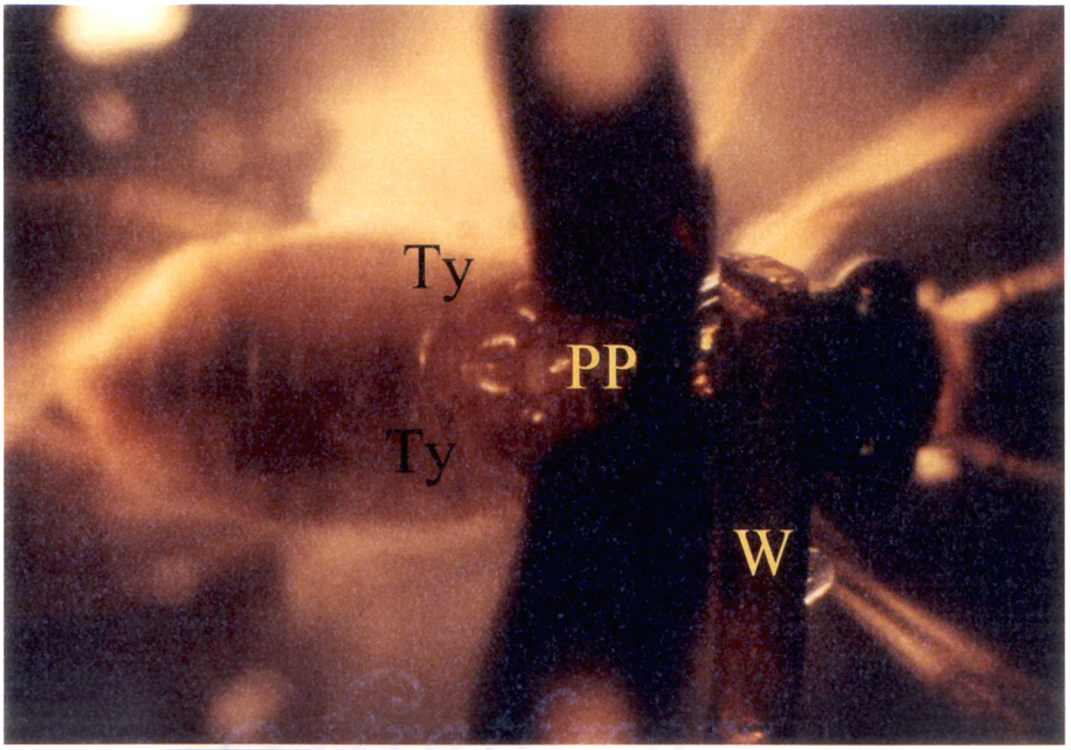

PP
Ty
2T
3T

Plate 9: Micrograph illustrating the tethered flight behavior of a live test subject. The wire tether is labeled (W). The pleural process is labeled (PP). The first abdominal tergum and tympanal regions are labeled (Ty). This photograph illustrates the unrestricting nature of the tether apparatus, which allows complete movement of the head, legs and abdomen as well as demonstration of the flight behavior. Flight was achieved by gently blowing on the insect. Stimulation with ultrasound caused an immediate cease of the flight behavior. 5X

Plate 10: Scanning electron micrograph of the tympanal region (Ty). Note the large size of the first abdominal tergum in comparison to the second abdominal tergum (2T) and pleural process (PP). The dimples in the tympana are the result of the dehydration process which causes the air-filled cavities of the tympanal organ to slightly collapse. 200X


Ty
PP
Ty
W

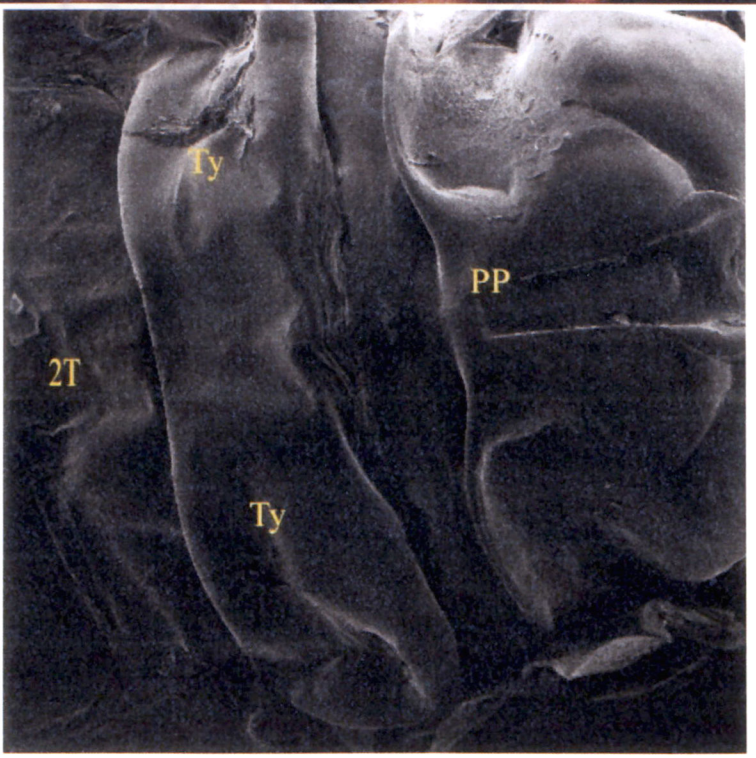
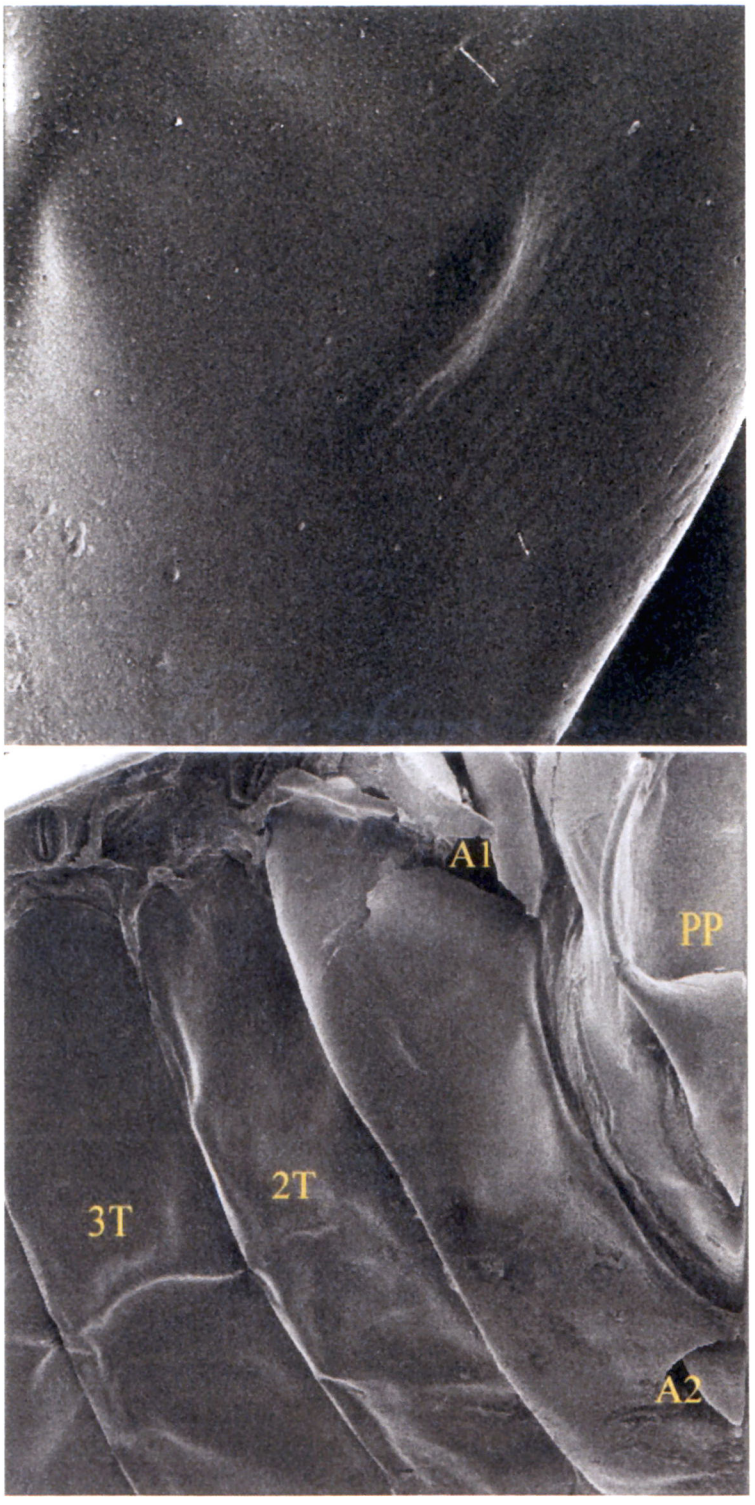

Ty
PP
2T
Ty

Plate 11: High magnification, Scanning electron micorgraph of the smooth surface of a tympanum. 880X

Plate 12: Scanning electron micrograph of the ablated tympanal regions of the 1st abdominal tergum of the test subject. The pleural process is labeled (PP) the second and third abdominal tergi are labeled 2T and 3T, respectively. The lower ablation (A1) was created by inserting a dissecting pin into the suspected region of the first abdominal tergum. Because of the size of the upper ablation (A2) it is likely that this ablation occurred as a result of removing the elytra and wings. 220X


A1
PP
2T
3T
A2

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